THE HUMAN GENE SLC25A17 ENCODES A PEROXISOMAL TRANSPORTER OF COENZYME A, FAD AND NAD⁺

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Short (page heading) title: SLC25A17 is a transporter of coenzyme A, FAD and NAD⁺ in peroxisomes

Synopsis
The essential cofactors coenzyme A (CoA), FAD and NAD⁺ are synthesized outside the peroxisomes and therefore must be transported into the peroxisomal matrix where they are required for important processes. In this work we have functionally identified and characterized SLC25A17, which is the only member of the mitochondrial carrier family that has previously been shown to be localized in the peroxisomal membrane. Herein, recombinant and purified SLC25A17 was reconstituted into liposomes. Its transport properties and kinetic parameters demonstrate that SLC25A17 is a transporter of CoA, FAD, FMN, AMP and to a lesser extent of NAD⁺, adenosine 3',5'-diphosphate (PAP) and ADP. SLC25A17 functioned almost exclusively by a counter-exchange mechanism, was saturable and inhibited by pyridoxal-5'-phosphate and other mitochondrial carrier inhibitors. It was expressed to various degrees in all the human tissues examined. Its main function is probably to transport free CoA, FAD and NAD⁺ into peroxisomes in exchange for intraperoxisomally generated PAP, FMN and AMP. This is the first report describing the identification and characterization of a transporter for multiple free cofactors in peroxisomes.

Keywords
Coenzyme A transporter, FAD transporter, NAD⁺ transporter, membrane transport, mitochondrial carrier, peroxisome

Abbreviations:
CoA, coenzyme A; FMN, flavin mononucleotide; NMN, nicotinamide mononucleotide; PAP, adenosine 3',5'-diphosphate.

INTRODUCTION
Peroxisomes are virtually ubiquitous organelles of eukaryotic cells that are involved in several metabolic pathways including fatty acid α- and β-oxidation, the biosynthesis of ether phospholipids and bile salts, and the catabolism of purines, polyamines and L-
pipecolic acid [1]. Although the peroxisomal membrane was originally thought to be freely permeable to solutes, various reports demonstrated the existence of peroxisomal transporters in both *Saccharomyces cerevisiae* and mammalian cells [2-6]. Moreover, a pH gradient between the cytosol and peroxisomal matrix was shown to exist, indicating that even the transport of protons between these two compartments is regulated [7-9]. Despite the considerable progress made in peroxisomal solute transport, as mentioned above, the function of many peroxisomal membrane proteins has yet to be determined. Furthermore, some of the already identified peroxisomal transporters are still relatively poorly characterized. For example, except for the yeast Pxa1p/Pxa2p and the human PMP70, which most likely transport long chain acyl-CoA esters, much remains to be determined about transported substrates and mechanisms of other peroxisomal ABC transporters [3].

Previously known as PMP34, SLC25A17 was first described by Wylin et al. [10] as a likely counterpart of the *Candida boidinii* PMP47 and by Honsho and Fujiki [11] as a model peroxisomal membrane protein to investigate topogenic signals. Both these studies demonstrated that SLC25A17 is localized to peroxisomes and belongs to the mitochondrial carrier family [10,11], in man known as the SLC25 solute carrier family [12,13]. Later, SLC25A17 was confirmed to be targeted to peroxisomes and found to possess some unquantified ATP transport activity [14]. Because this latter study reported only the percentage of radioactivity, and not the amount of [14C]ATP taken up by liposomes reconstituted with SLC25A17, and employed a limited set of nucleotides, we set out to perform a more in-depth study on the activity of SLC25A17.

In this study we provide direct evidence that SLC25A17 is a transporter for CoA, FAD and to a lesser extent NAD\(^+\). SLC25A17 was expressed in *Escherichia coli*, purified, reconstituted into liposomes, and shown to transport CoA, FAD, NAD\(^+\), adenosine 3',5'-diphosphate (PAP), FMN, AMP and ADP, but not many other compounds. The transporter nearly exclusively catalyzed a counter-exchange of substrates, was saturable and inhibited by pyridoxal-5'-phosphate and other inhibitors; its mRNA was found in all tissues investigated. This is the first time that a carrier for multiple free cofactors has been identified and characterized at the molecular level.

**EXPERIMENTAL**

**Construction of expression plasmid**

The coding sequence for SLC25A17 (NM_006358) was amplified from HepG2 cell cDNA by PCR using primers corresponding to the extremities of the coding sequence with additional BamHI and XhoI sites. The amplified product was cloned into the pET-21b expression vector. Transformants of *E. coli* TOP10 cells were selected on 2X TY plates containing ampicillin (100 μg/ml) and screened by direct colony PCR and restriction digestion of plasmids (1 x TY is 16 g/l tryptone, 10 g/l yeast extract and 5 g/l NaCl, pH 7.4). The sequence of the insert was verified.

**Bacterial expression and purification of SLC25A17**

SLC25A17 was produced as inclusion bodies in the cytosol of *E. coli* as described previously [15], except that the host cells were *E. coli* strain Rosetta gami B (Novagen). Control cultures with the empty vector were processed in parallel. Inclusion bodies were purified on a sucrose density gradient [15], washed at 4°C with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 6.5), then twice with a buffer containing Triton X-114 (3%, w/v), 1 mM EDTA and 10 mM PIPES-NaOH, pH 7.0, and once again with TE buffer. SLC25A17 was solubilized in 1.7% sarkosyl (w/v), and a small residue was removed by centrifugation (258000 x g, 1 h). In some experiments, SLC25A17 with a C-terminal His\(_6\)-tag was purified
from *S. cerevisiae* ant1Δ cells transformed with a single copy vector containing the SLC25A17-His6 downstream of the catalase A promoter, exactly as described by Visser et al. [14].

**Reconstitution of SLC25A17 into liposomes and transport assays**

The recombinant protein in sarkosyl (N-dodecanoyl-N-methyl-glycine sodium salt) was reconstituted into liposomes in the presence and absence of substrates as described before [16]. External substrate was removed from proteoliposomes on Sephadex G-75 columns, pre-equilibrated with 50 mM NaCl and 10 mM MOPS-NaOH at pH 7.4 (buffer A). Transport at 25°C was started by adding [14C]AMP (ARC), [14C]ADP (Perkin Elmer), [14C]ATP (Hartman Analytic), [3H]NAD+ (Perkin Elmer), FAD, FMN or the indicated substrates to proteoliposomes and terminated by addition of 10 mM pyridoxal-5’-phosphate and 10 mM bathophenanthroline (the “inhibitor stop” method [16]). In controls, inhibitors were added at the beginning together with the substrate. The external substrate was removed, and the entrapped substrate was measured [16]. The experimental values were corrected by subtracting control values. The initial transport rate was calculated from the substrate taken up by proteoliposomes after 2 min (in the initial linear range of substrate uptake). In some experiments the uptake of 40 μM [14C]ATP into proteoliposomes loaded with 20 mM ATP was measured after 15 min incubation as described by Visser et al. [14]. For efflux measurements i) proteoliposomes containing 2 mM FAD were passed through Sephadex G-75 to remove the external substrate, and ii) proteoliposomes containing 2 mM AMP were labeled with 5 μM [14C]AMP by carrier-mediated exchange equilibration for 40 min [16] and then passed through Sephadex G-75. Efflux was started by adding unlabeled external substrate in buffer A or buffer A alone to aliquots of proteoliposomes and terminated by adding the inhibitors indicated above.

**Expression analysis by real-time PCR**

FirstChoice™ total RNAs from human tissues (Ambion) were reverse-transcribed with the GeneAmp RNA PCR Core kit with random hexamers as primers. Real Time PCRs were conducted as described previously [17] using a TaqMan-MGB probe and primers based on the coding sequence of SLC25A17 (Assay ID: Hs00197168_m1, Applied Biosystems). To correct for differences in the amount of starting first-stranded cDNAs, the human peptidylprolyl isomerase A was amplified in parallel as a reference gene.

**Other methods**

Proteins were analyzed by SDS-PAGE and stained with Coomassie Blue dye. The identity of purified SLC25A17 was assessed by matrix-assisted laser desorption ionization-time of flight mass spectrometry of trypsin digests of the corresponding band excised from a Coomassie-stained gel [18]. The amount of pure SLC25A17 was estimated by laser densitometry of stained samples, using carbonic anhydrase as protein standard [17]. The share of protein incorporated into liposomes measured as before [17] was about 20% of the protein added to the reconstitution mixture. FAD and FMN were measured fluorimetrically by a LS 50B Fluorescence Spectrometer (PerkinElmer) at 450-nm excitation and 530-nm emission wavelengths [19]; all data were corrected by subtracting the background fluorescence.

**RESULTS**

**Bacterial expression of SLC25A17**
The SLC25A17 gene was expressed in *E. coli* Rosetta gami B(DE3) (Fig. 1). The gene product accumulated as inclusion bodies and was purified on a sucrose discontinuous gradient followed by centrifugation and washing (Figure 1, lane 5). The identity of the purified protein was confirmed by mass spectrometry analysis of trypsin digests. The protein was not detected in bacteria harvested immediately before induction of expression (Figure 1, lane 2), nor in cells harvested after induction but lacking the coding sequence in the expression vector (Figure 1, lane 3). Approximately 10 mg of purified protein per liter of culture was obtained.

**Functional characterization of SLC25A17**

The recombinant purified SLC25A17 was reconstituted into liposomes and its transport activity for potential substrates was tested in homo-exchange experiments (i.e. with the same substrate inside and outside). Using external and internal substrate concentrations of 0.1 and 10 mM, respectively, the reconstituted protein catalyzed $^{14}$C$\text{AMP}/\text{AMP}$ and $^{14}$C$\text{ADP}/\text{ADP}$ exchanges that were inhibited completely by a mixture of pyridoxal-5'-phosphate and bathophenanthroline. By contrast, despite the long incubation period (i.e. 30 min), virtually no homo-exchange activity was observed for ATP, GTP, TTP, CTP, phosphate, malate, citrate, glutamate, glutamine, ornithine, arginine and S-adenosylmethionine (results not shown). Furthermore, no $^{14}$C$\text{AMP}/\text{AMP}$ exchange activity was detected when SLC25A17 had been boiled before incorporation into liposomes or if proteoliposomes were reconstituted with sarkosyl-solubilized material from bacterial cells either lacking the expression vector for SLC25A17 or harvested immediately before induction of expression.

To further investigate the substrate specificity of SLC25A17 the initial rate of $^{14}$C$\text{AMP}$ uptake into proteoliposomes that had been preloaded with a variety of potential substrates was measured (Figure 2). The highest activity of $^{14}$C$\text{AMP}$ uptake into proteoliposomes was observed with internal AMP, CoA, dephospho-CoA and FAD. To a lesser extent, $^{14}$C$\text{AMP}$ also exchanged with internal acetyl-CoA, FMN, ADP, PAP and NAD$^+$. In contrast, a marginal exchange of $^{14}$C$\text{AMP}$ was observed with internal 3'-AMP, cAMP, ATP, propionyl-CoA, NADH, NADP$^+$, NADPH, NMN, GMP, IMP, CMP, TMP, UMP, phosphate, pyrophosphate, thiamine, thiamine pyrophosphate, pantothenate (Figure 2) and GTP, TTP, CTP, malate, citrate, glutamate, glutamine, ornithine, arginine and S-adenosylmethionine (data not shown). The residual activity in the presence of these compounds was approximately the same as that observed in the presence of NaCl (Figure 2). In addition, no uptake of $^{14}$C$\text{AMP}$ into pure liposomes, e.g. without incorporated protein, was observed (data not shown).

In another set of experiments we measured the uptake of the fluorescent FAD and FMN and of the radioactively labeled NAD$^+$ and ADP into proteoliposomes in the presence and absence of internal substrate. The results of Fig. 3 demonstrate that FAD and FMN were taken up by liposomes reconstituted with SLC25A17 in exchange with internal AMP, CoA or PAP, and $^3$H$\text{NAD}^+$ and $^{14}$C$\text{ADP}$ with internal AMP, FAD, FMN, CoA or PAP to a considerable extent. By contrast, the uptake of FAD, FMN, NAD$^+$ or ADP into proteoliposomes containing no substrate (NaCl present) was very low (Figure 3); the uptake of $^{14}$C$\text{ATP}$ and $^{14}$C$\text{CTP}$ in the presence of internal FAD, FMN, NAD$^+$ or ADP was negligible; and finally, the uptake of FAD, FMN, NAD$^+$, AMP or ADP into pure liposomes was virtually null, despite the long incubation period (i.e. 30 min) (results not shown). Therefore, in the presence of a suitable counter-substrate FAD, FMN, NAD$^+$ and ADP are transported by reconstituted SLC25A17 not only when present inside proteoliposomes but also when added externally.
Given that SLC25A17 (PMP34) was proposed to be an ATP transporter [14] in contrast to the data reported above, we measured the uptake of \[^{14}C\]ATP in proteoliposomes using the same experimental conditions employed in ref. 14. In these experiments we found an extremely low uptake of \[^{14}C\]ATP, equal to 0.73 ± 0.2 μmol / 15 min / g protein, into proteoliposomes containing ATP. Therefore, ATP is virtually not transported by SLC25A17.

The effect of inhibitors on the \[^{14}C\]AMP/AMP exchange reaction catalyzed by reconstituted SLC25A17 was also examined (Figure 4). The exchange reaction was inhibited strongly by pyridoxal-5'-phosphate, bathophenanthroline, \(\text{HgCl}_2\) and mersalyl, and partially by \(\alpha\)-cyano-4-cinnamate and \(\rho\)-hydroxymercuribenzoate. In contrast, little effect was observed with \(\text{N-ethylmaleimide, butylmalonate, 1,2,3-benzenetricarboxylate, carboxyatractyloside and bongkrekic acid, which are known inhibitors of other mitochondrial carriers (Figure 4).}

**Kinetic characteristics of recombinant SLC25A17**

In Figure 5A the time-course is compared for the uptake of 1 mM \[^{14}C\]AMP into reconstituted liposomes measured either as uniport (in the absence of internal substrate) or as exchange (in the presence of one of the indicated substrates). The uptake of \[^{14}C\]AMP by exchange with internal CoA, FAD, PAP or FMN substantially increased with time, equilibrium being approached at or after 60 min incubation. In comparison, the uniport uptake of \[^{14}C\]AMP was very low (Figure 5A). The efflux of \[^{14}C\]AMP from prelabeled active proteoliposomes was investigated in figure 5B. In the absence of external substrate a marginal efflux was observed. However, upon addition of external AMP, PAP, CoA or FAD a substantial efflux of radioactivity occurred which was prevented by the inhibitors pyridoxal-5'-phosphate and bathophenanthroline. Also the efflux of fluorescence from proteoliposomes preloaded with FAD was pronounced upon addition of CoA or AMP to the reaction medium, whereas it was much lower and slower upon addition of buffer alone, i.e. in the absence of externally added substrates (Figure 6). In control assays, there was no FAD efflux when transport inhibitors were added at time zero. These results indicate that, at least under the experimental conditions used, reconstituted SLC25A17 functions essentially by a counter-exchange mechanism.

The kinetic constants of recombinant SLC25A17 were determined by measuring the initial transport rate at various external \[^{14}C\]AMP concentrations in the presence of a fixed saturating internal concentration of 10 mM AMP. The transport affinity (Km) and specific activity (Vmax) values for \[^{14}C\]AMP/AMP exchange at 25°C were 0.19 ± 0.03 mM and 74 ± 9 μmol/min/g protein, respectively. Several external substrates were competitive inhibitors of \[^{14}C\]AMP uptake as they increased the apparent Km without changing the Vmax (not shown). The Ki values of these substrates for SLC25A17 were the following: CoA 19.6 ± 3.8 μM, dephospho-CoA 14.4 ± 5.4 μM, FAD 2.6 ± 1.3 μM, FMN 10.2 ± 3.3 μM, NAD⁺ 0.85 ± 0.12 mM, PAP 0.78 ± 0.1 mM and ADP 0.6 ± 0.08 mM.

**Expression of the SLC25A17 gene in human tissues**

The tissue distribution of SLC25A17 mRNA was determined by real-time PCR. The data of Figure 7 show that the SLC25A17 gene was expressed in all 21 human tissues investigated. This finding is in line with the fact that peroxisomes are present in virtually all eukaryotic cells except in erythrocytes and male germ cells [20]. It should be noted that as post-transcriptional mechanisms may operate, the levels of expression presented in Figure 7 do not necessarily reflect the ratios of transport activities.
DISCUSSION

In this work, the function of SLC25A17 was investigated by direct transport assays upon expression in E. coli, purification and incorporation of the protein into phospholipid vesicles. The transport properties and kinetic characteristics of recombinant SLC25A17, reported here, unequivocally demonstrate that this protein is a transporter of CoA, FAD and to a lesser extent NAD⁺. Given that the subcellular localization of SLC25A17 to peroxisomes has been proven by three different groups [10,11,14], this is the first time that a peroxisomal carrier for the free cofactors CoA, FAD and NAD⁺ has been identified from any organism. Of note, the specific activity of reconstituted SLC25A17 is comparable with the activities of other SLC25 proteins [15,17,21-24], and the Ki values of CoA, FAD and NAD⁺ for SLC25A17 are lower or not much higher than the cytosolic-free concentrations of these cofactors [25-27].

Besides CoA, FAD and NAD⁺, SLC25A17 transports AMP, dephospho-CoA, acetyl-CoA, FMN, ADP and PAP. Because in humans the biosynthesis of CoA, FAD and NAD⁺ takes place entirely outside the peroxisomes, a primary function of SLC25A17 is to catalyze the import of CoA, FAD and NAD⁺ into the peroxisomal matrix where they are indispensable for the activity of various intraperoxisomal enzymes. Moreover, because SLC25A17 functions almost exclusively by a counter-exchange mechanism, the carrier-mediated uptake of the free cofactors CoA, FAD and NAD⁺ requires efflux of a counter-substrate. Our transport measurements in reconstituted liposomes indicate that the most likely candidates to exchange with external CoA, FAD and NAD⁺ are PAP, FMN, AMP and ADP, which are effective reactants of SLC25A17. Thus, in the intraperoxisomal matrix PAP is generated from CoA and CoA derivatives by the Nudix hydrolases NUDT7 and NUDT19 [28-30], FMN is generated from FAD by NUDT12 [31], AMP from ATP by acetyl-CoA synthetases and from NAD(H) by NUDT12 [32] and ADP from ATP by PsLon, an ATP-dependent protease [33]. Our results do not agree with the previous conclusion that SLC25A17 (PMP34) is an ATP transporter [14]. This discrepancy cannot be attributed to methodological differences because, using the same experimental conditions employed in ref. 14, we did not detect any significant ATP transport. Furthermore, in this work we have shown that SLC25A17 does not significantly catalyze any of the [¹⁴C]AMP/ATP, [¹⁴C]ATP/ATP, [¹⁴C]ATP/FAD, [¹⁴C]ATP/NAD⁺ and [¹⁴C]ATP/ADP exchanges. It is worth mentioning that the authors of the previous investigation [14] reported only the percentage of radioactivity without giving numbers, i.e. not the counts of radioactivity per 15 min and the specific radioactivity (counts of radioactivity / 15 min / nmol of [¹⁴C]ATP). Therefore, it is impossible to calculate the extent of ATP transport activity from their results [14].

In a phylogenetic tree of H. sapiens, S. cerevisiae and A. thaliana mitochondrial carriers (see Figure 3A of ref. 13), SLC25A17 clusters with the carriers for NAD⁺ [23, 34], pyrimidine nucleotides [35,36], FAD/folate [37-39], the peroxisomal Ant1p [40] and its homologs At3g05290 and At5g27520 [41], and the peroxisomal At2g39970 (see below). With all these transporters, except yeast Ant1p and its homologs in Arabidopsis, SLC25A17 shares the distinct feature of having a tryptophan, instead of an acidic residue, in the mitochondrial carrier signature motif PX(D/E)XX(R/K) present in the second repeat of these proteins (see Figure 2 of ref. 13). Nevertheless, the substrate specificity of SLC25A17 is distinct from that of any other carrier present in the above mentioned cluster, although some overlapping functions (i.e. substrates transported) occur.

During the revision of this manuscript two papers have been published on the closest relative of SLC25A17 in Arabidopsis, i.e. the peroxisomal membrane protein encoded by At2g39970, also known as PMP38. Importantly, in the first paper it was found...
that PMP38 transports NAD⁺, AMP and ADP but not ATP [42]. In the second paper, in which the APEM3 gene encoding PMP38 was knocked out, the authors concluded that this protein plays an important role in peroxisomal proliferation and stated that they were unable to detect transport of ATP catalyzed by PMP38 [43]. It should be added that PMP38, i.e. At2g39970, was previously found to be incapable of complementing the growth defect of ant1 null strain, whereas At3g05290 and At5g27520 exhibited this ability [41]. It is also interesting to recall that the yeast NAD⁺ carrier [23] and Arabidopsis mitochondrial and plastidial NAD⁺ carriers [34] transport FAD and FMN as well, although to a much lesser extent than NAD⁺, AMP and GMP.

The closest relative of SLC25A17 in yeast, Ant1p, was found to transport ATP, AMP and ADP with high efficiency and to a lesser extent UDP, UTP, CDP and CTP [40]. Unfortunately, most of the substrates found to be transported by SLC25A17 herein, i.e. FAD, FMN, CoA, NAD⁺ and PAP, were not tested in this early study. However, there are some important sequence differences between SLC25A17 and Ant1p. Apart from the presence of a tryptophan in the signature motif of the second repeat in SLC25A17 and not in Ant1p, both proteins share 26% identical amino acids; this value is too low to draw definite conclusions about the substrate specificity of mitochondrial carrier family members. Furthermore, the residues of the even-numbered transmembrane α-helices, which have been proposed to form the similarly located substrate binding site of mitochondrial carriers [44] markedly differ between SLC25A17 (S74 and S78 in H2, L175 and V176 in H4 and Q276 in H6) and Ant1p (A83 and Q87 in H2, L191 and T192 in H4 and K286 in H6). All these differences between SLC25A17 and Ant1p suggest that they may display differences in the substrates they transport. In general, low homology between mitochondrial carriers, as found in the closest relatives of distant organisms, should be regarded with caution when drawing conclusions about their substrate specificity in the absence of direct transport assays involving a wide range of potential substrates. A clear example is provided by the closest S. cerevisiae sequence to the mammalian oxoglutarate carrier, namely the YLR348c gene product, which was identified as the yeast dicarboxylate carrier from its transport characteristics in reconstituted liposomes [45]. Furthermore, disagreement between the predicted and experimentally tested functions of several Arabidopsis mitochondrial carriers has recently been pointed out [13]. Even identified isoforms of mitochondrial carriers with a high percentage of identical amino acids may display clear differences in substrate specificity. For example, ORC1 (i.e. human ornithine carrier 1) transports the L-forms of ornithine, lysine and arginine, whereas ORC2 has a broader specificity also transporting the D-forms of these amino acids as well as histidine and homoarginine, despite the fact that they share 87% identical amino acids [46]. Historically, the first mitochondrial carrier found to be localized in the peroxisomal membrane is PMP47 of Candida boidinii [47]. Nothing is known about the substrate(s) transported by PMP47, although it was speculated to be involved in ATP transport without any direct empirical evidence [48].

Apart from the relatively low homology that SLC25A17 displays with the transporters discussed above (23-31% identical amino acids), SLC25A17 does not exhibit significant sequence homology with any other known carrier greater than the homology existing among the different members of the mitochondrial carrier family. However, several protein sequences available in databases are likely to be orthologs of SLC25A17 in other organisms. These sequences include: XP_531726.1 from C. familiaris (97% identity), NP_001119741.1 from R. norvegicus (95%), NP_001039413.1 from B. taurus (94%), NP_035529.1 from M. musculus (94%), NP_001088333.1 from X. laevis (73%), NP_001092731.1 from D. rerio (72%), XP_623636.1 from A. mellifera (47%), NP_728982.1 from D. melanogaster (43%), and XP_001662175.1 from A. aegypti (46%). To our knowledge, none of these proteins has been characterized biochemically. Future
studies are warranted to investigate the effects of SLC25A17 knock-down by RNAi or SLC25A17 knock-out mice, and to ascertain a potential regulatory role of SLC25A17 in peroxisomal function as well as its involvement in peroxisome-related disorders.

FUNDING

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REFERENCES


Legends to figures

Figure 1. Expression in E. coli and purification of SLC25A17. Proteins were separated by SDS-PAGE and stained with Coomassie Blue. Markers in the left-hand column (bovine serum albumin, carbonic anhydrase and cytochrome c); lanes 1-4, E. coli Rosetta gami B(DE3) containing the expression vector without (lanes 1 and 3) and with (lanes 2 and 4) the coding sequence of SLC25A17. Samples were taken at the time of induction (lanes 1 and 2) and 4 h later (lanes 3 and 4). The same number of bacteria was analyzed in each sample. Lane 5, purified SLC25A17 protein (6 μg) derived from bacteria shown in lane 4.

Figure 2. Substrate specificity of SLC25A17. Liposomes reconstituted with SLC25A17 were preloaded internally with various substrates (concentration 5 mM). Transport was started by addition of 0.2 mM [14C]AMP and terminated after 2 min. Values are means ± S.E.M. of at least three duplicate independent experiments. Actyl-CoA, acetyl-CoA; Ppynyl-CoA, propionyl-CoA; Pi, phosphate; PPi, pyrophosphate; Th, thiamine; ThPP, thiamine pyrophosphate; Pan, pantothenic acid.

Figure 3. Uptake of FAD, FMN, NAD+ and ADP into liposomes reconstituted with SLC25A17. Proteoliposomes were preloaded internally with various substrates or NaCl (concentration 5 mM). Transport was initiated by adding 0.85 mM [14C]ADP, 0.85 mM [3H]NAD+, 40 μM FAD or 200 μM FMN and terminated after 30 min. Values are means ± S.E.M. of at least three duplicate independent experiments.

Figure 4. Effect of inhibitors on the [14C]AMP/AMP exchange by SLC25A17. Proteoliposomes were preloaded internally with 5 mM AMP and transport was initiated by adding 0.2 mM [14C]AMP to liposomes reconstituted with SLC25A17. The incubation time was 2 min. Thiol reagents and α-cyanocinnamate were added 2 min before the labeled substrate. The final concentrations of the inhibitors were 10 μM (CAT, carboxyatractyloside; BKA, bongkrekic acid), 10 mM (PLP, pyridoxal-5’-phosphate; BAT, bathophenanthroline), 0.2 mM (pHMB, p-hydroxymercuribenzoate; MER, mersalyl), 1 mM (NEM, N-ethylmaleimide; CCN, α-cyanocinnamate) and 2 mM (BMA, butylmalonate; BTA, 1,2,3-benzenetricarboxylate). Values are extents of inhibition (%) ± S.E.M. of at least three duplicate independent experiments.

Figure 5. Kinetics of [14C]AMP transport into proteoliposome reconstituted with SLC25A17. (A) Uptake of AMP. 1 mM [14C]AMP was added to liposomes reconstituted with SLC25A17 and preloaded internally with 10 mM AMP (●), CoA (♦), FAD (○), PAP (▲), FMN (▼), NAD+ (▲), NMN (□) or NaCl (■). Additionally, 1 mM [14C]AMP was added to pure liposomes preloaded internally with 10 mM AMP (X). (B) Efflux of [14C]AMP. The efflux of [14C]AMP was started by adding buffer A alone (■), AMP (●), 5 mM CoA (♦), FAD (○), PAP (▲), NAD+ (▲), FMN (▼) or CoA, 10 mM pyridoxal-5’-phosphate and 10 mM bathophenanthroline (○) in buffer A. Values are means of at least three duplicate independent experiments. S.E.M. of all the values are ≤ 10%. In (A) differences between all the values with internal AMP, FAD and PAP, with internal CoA from 10 to 60 min, and with internal FMN at 30 and 60 min and controls (samples with internal NaCl) were significant (P < 0.01, one way ANOVA followed by the Bonferroni t-test). In (B) differences...
between all the values with added AMP and PAP, with added CoA from 10 to 40 min and with added FAD at 20 and 40 min, and controls (with added inhibitors) are significant (P < 0.01, one way ANOVA followed by the Bonferroni t-test).

**Figure 6. Efflux of FAD from liposomes reconstituted with SLC25A17.** The efflux was started by adding buffer A alone (▲), 5 mM CoA (■), 5 mM AMP (●) or 10 mM pyridoxal-5'-phosphate and 10 mM bathophenanthroline (X). Values are means ± S.E.M. of four duplicate independent experiments.

**Figure 7. Expression of SLC25A17 in human tissues.** The relative quantification of SLC25A17 mRNA in the indicated tissues was performed according to the comparative method (2^(-△Ct)). The liver Ct for SLC25A17 was used as internal calibrator. For the internal calibrator, the △Ct equals zero and 2^0 equals one. For the remaining tissues, the values 2^(-△Ct) on the abscissa indicate the fold change in gene expression relative to liver. Values are means ± S.D. of three duplicate independent experiments.
Figure 2

The figure shows the enzymatic activity in micromoles per minute per gram of protein for various substrates and coenzymes. The substrates include NaCl, Pan, ThPP, Th, PPI, Pi, UMP, TMP, CMP, IMP, GMP, NMN, NADPH, NADP^+, NADH, NAD^+, ATP, PAP, ADP, FMN, FAD, Ppynyl-CoA, Actyl-CoA, dPCoA, CoA, cAMP, 3'-AMP, and AMP. The activity is measured in micromoles per minute per gram of protein (µmol/min/g protein).
Figure 3

This figure shows the concentration of various molecules in the biochemical reaction. The molecules include NaCl, PAP, CoA, FMN, FAD, AMP, ADP, NAD⁺, and FMN. The x-axis represents the concentration of these molecules, measured in μmol/30 min/g prot. The y-axis lists the molecules, and the bars indicate the concentration levels.

The concentrations for each molecule are as follows:
- NaCl
- PAP
- CoA
- FMN
- FAD
- AMP
- ADP
- NAD⁺
- FMN
- FAD

The data is presented in a bar chart format, with error bars indicating variability or standard deviation.
Figure 4

The figure shows the percentage inhibition of various compounds and combinations on the biochemical activity. The compounds include BKA, CAT, CCN, MER, HgCl$_2$, NEM, pHMB, BMA, BTA, BAT, PLP, and BAT + PLP. The inhibition is measured on a scale from 0 to 100%.
Figure 5

(A) Efflux of [14C]AMP (cpm x 10^3) from cells incubated with different concentrations of substance A.

(B) Efflux of [14C]AMP (cpm x 10^3) from cells incubated with different concentrations of substance B.

(time (min))
Figure 6

Efflux of FAD (μmol / g prot)

Time (min)
Figure 7

The figure shows a bar chart representing the expression levels of different tissues. The x-axis is labeled with the symbols $2^{-\Delta\Delta Ct}$, indicating a measure of expression fold change. The y-axis lists various tissues, including:

- Adipose
- Bladder
- Brain
- Cervix
- Colon
- Esophagus
- Heart
- Kidney
- Liver
- Lung
- Ovary
- Pancreas
- Placenta
- Prostate
- Skeletal Muscle
- Small Intestine
- Spleen
- Testis
- Thymus
- Thyroid
- Tracheas

Each tissue is represented by a bar indicating its expression level. The bars are not labeled with specific values, but the relative heights suggest differences in expression across the tissues.